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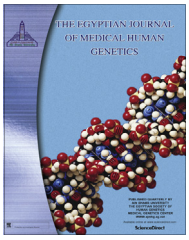

ORIGINAL ARTICLE

Association between methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism and risk of ischemic stroke in North Indian population: A hospital based case–control study

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KEYWORDS

Ischemic stroke;
Polymorphisms;
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North India;
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Abstract *Objective:* The aim of the present case–control study was to determine the association between methylene tetrahydrofolate reductase (MTHFR) C677T (rs1801133) gene polymorphism and risk of ischemic stroke (IS) in North Indian population.

Methods: Patients with IS and age-sex matched controls were recruited from Neurology Outpatient Department and Ward of All India Institute of Medical Sciences, New Delhi, India. Genotyping was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. PCR–RFLP results of nine randomly selected samples were confirmed by DNA sequencing. Genotypic and allelic distributions were compared between cases and controls. Statistical analysis was done by STATA, version 13.0 software.

Results: Hypertension, diabetes, dyslipidemia, low socioeconomic status and family history of stroke were found to have an independent association with the risk of IS after adjusting for potential confounding factors. Mean age of cases and controls were 52.83 ± 12.59 and 50.97 ± 12.70 years. Multivariate logistic regression analysis showed an independent association between MTHFR C677T gene polymorphism and risk of IS (OR 1.91; 95% CI 1.07–3.41; $p = 0.028$) under dominant model [CT + TT vs. CC]. MTHFR C677T gene polymorphism was found to be independently associated with risk of small vessel disease (SVD) after adjustment for potential confounding factors [OR 2.51; 95% CI 1.30–4.85; $p = 0.006$] under the dominant model.

Conclusion: Findings of the present study suggest that MTHFR C677T gene polymorphism might be a risk factor of IS mainly for SVD subtypes of IS in North Indian population. Further large prospective studies are required to confirm these findings.

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1. Introduction

Stroke is a complex multi-factorial, polygenic disease resulting from a combination of environmental, lifestyle and genetic risk factors [1,2]. Approximately 80–90% of strokes are ischemic in origin. The role of genetic determinants in ischemic stroke (IS) has been demonstrated by a number of studies which include animal model, twin and family studies [3]. Despite intensive research efforts, the genomic etiology of IS remains elusive. Hyperhomocysteinemia is reported to have an independent association for the risk of vascular events including stroke [4]. Among the genes involved in the metabolism of homocysteine (Hcy), methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism plays a pivotal role by decreasing the activity of MTHFR and increasing Hcy levels [5,6]. Human MTHFR gene is located on chromosome 1p36.3 [7].

MTHFR is a key enzyme in homocysteine metabolism which catalyzes irreversibly conversion of 5,10-methyl enetetrahydrofolate to 5-methylenetetrahydrofolate, which is the main circulatory form of folate. This product provides methyl group in the remethylation reaction of homocysteine to methionine [8], which in turn is required for the synthesis of S-adenosyl methionine (SAM) through methionine cycle [9]. SAM is a primary methyl group donor for most of the biological methylation reactions [10,11]. A single base pair (677C/T) transition in the MTHFR gene changes the alanine to valine amino acid residue which influences enzyme thermolability with 30% decreased enzyme activity [12] and, in turn resulting in elevated level of homocysteine and hypomethylation in homozygous state [13]. Several studies have shown that an elevated level of homocysteine leads to subsequent increased likelihood of stroke [14]. A recent study has suggested that hyperhomocysteinemia is associated with SVD and LVD subtype of stroke [15]. The MTHFR C677T polymorphism is suspected to induce hypomethylation which has been found to be significantly associated with increased susceptibility of IS [16].

Several studies have examined the association between MTHFR C677T gene polymorphism and risk of IS, however, results of these studies are inconsistent. Some reports have shown relationship between MTHFR C677T polymorphism and risk of IS while others have failed to replicate such association [17]. Four studies with small sample size have been reported from Indian population out of which three were found to have a significant association with the risk of IS [18–20], while one did not find significant association between MTHFR C677T polymorphism and risk of IS [21]. To draw a more profound conclusion, the present case–control study was undertaken to determine the association of MTHFR C677T (rs1801133) gene polymorphism and susceptibility to IS in North Indian population.

2. Methods

2.1. Study participants

The study was conducted in the Department of Neurology, All India Institute of Medical Sciences (AIIMS), New Delhi. It was a hospital based case–control study and was completed in 2 years (November 2012 to October 2014). A total of 450 patients having stroke symptoms were screened. Patients with a history of transient ischemic attack, fever, rheumatologic disease, autoimmune disease, any acute or chronic infection, computed tomography (CT) or magnetic resonance imaging (MRI) proven hemorrhagic stroke, and a history of regular immunosuppressive or analgesic therapies were excluded. A total of 250 patients were recruited for the study after radiological confirmation of IS by CT or MRI scans of the brain.

All the patients had clinical signs which were in concordance with the World Health Organization definition of stroke. The control group comprised of 250 age and sex matched healthy individuals which mainly consisted of volunteers and healthy people accompanying the patients in the general outpatient department (OPD) and were assessed by questionnaire for verifying stroke free status (QVFSS) [22]. Informed consent was obtained from all the subjects before collecting the information. The study was approved by the Local Institutional Ethics Committee. The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

2.2. Clinical examination

A detailed history and clinical evaluation were carried out. The assessment of patients was done on the day of admission and 6 months after the admission. Stroke was classified using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [23]. The National Institute of Health Stroke Scale (NIHSS) was used on the day of admission for the determination of clinical severity. Definitions of variables were same as present in our published study protocol [24].

2.3. Laboratory investigations

Four milliliter (ml) blood samples were taken in an ethylene diamine tetra acetic acid (EDTA) vial from patients and controls. Total genomic DNA was isolated from whole blood through standard phenol–chloroform method.

2.4. Genotype determination

The genotypes of MTHFR C677T gene were detected by using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique. The primers were designed for the Single Nucleotide Polymorphism (SNP) using Primer3 online tool, (http://bioinfo.ut.ee/primer3-0.4.0/). The MTHFR C677T region was genotyped as previously described [25]. Forward 5-GCACTTGAAGAGAAGGTGTC-3 and reverse 5-AGGACGGTGCGGTGAGAGTG-3 primers in T-100 thermal cycler (Bio-Rad). The primers were synthesized by Imperial Sciences (Gurgaon, India) under standard conditions. The conditions for amplification were as follows: Initial melting step of 5 min at 94 °C, followed by 35 cycles of 45 s denaturation at 94 °C, 45 s annealing at 65 °C, 1 min extension at 72 °C, and a final elongation step of 10 min at 72 °C. Genotyping was performed by digesting the PCR products by using *Hinf I* restriction enzyme (TAKARA BIO INC, Japan; Code No. 1238A) and incubating at 37 °C overnight. After amplification and digestion, the products were confirmed by agarose gel electrophoresis. The bands of 198 bp and 175 bp were observed after digestion as seen through the UV

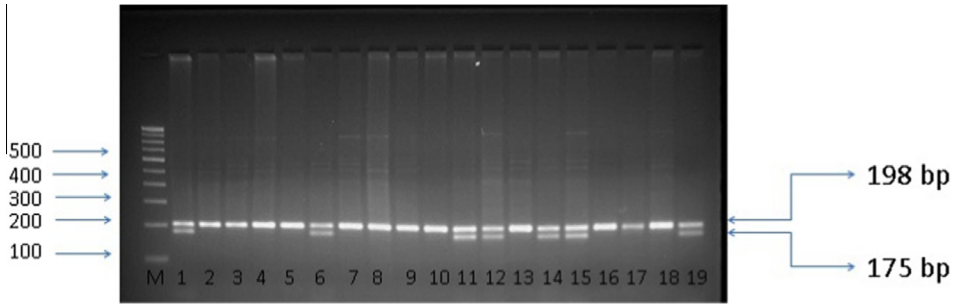

Figure 1 Gel image of PCR-RFLP of MTHFR C677T gene polymorphism. Loading sequence: 100 bp ladder. Lane 2–5, 7–10, 13, 16–18: C/C genotype (wild type). Lane 1, 6, 10–12, 14, 15 and 19: C/T genotype (heterozygous).

transilluminator. A single band of 198 bp represented CC (wild type) genotype; two bands of 198 bp and 175 bp represented CT (heterozygous) genotype and a single band of 175 bp represented TT (polymorphic) genotype (Fig. 1).

2.5. Statistical analysis

The chi-square test was used to determine whether the allelic frequencies were in accordance with Hardy–Weinberg equilibrium (HWE) or not. The conditional logistic regression analysis was used to estimate odds ratio (OR) and 95% confidence intervals (CIs) for the strength of association between MTHFR gene polymorphism and the risk of IS. Multivariate logistic regression was used to control the confounding effect of demographic and risk factor variables. Tests were considered statistically significant at $p < 0.05$. Data were analyzed using the STATA, version 13.0 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

2.6. Sample size calculation

Sample size was calculated by STATA version 13.0 software. Sample size for this study was calculated by using the frequencies of cases and controls of two published studies from Indian population in which association between MTHFR C677T polymorphism and risk of IS was reported [18–19]. The pooled frequency of exposure was 0.01 in controls and 0.04 in cases. With 80% power and 5% alpha, sample size obtained was 207 cases and 207 controls. We have taken a final sample size of 250 cases and 250 controls for the present study, keeping in mind the possibility of any sample loss.

3. Results

Two-hundred fifty IS cases and two-hundred fifty age-sex matched controls were included in the study. The mean age of IS patients was 52.83 ± 12.59 years, in the control group was 50.97 ± 12.70 years and both groups consisted of 203 males and 47 females. There was no statistical difference between the age of cases and controls ($p < 0.05$). The clinical characteristics and demographic variables of IS patients and controls are presented in Tables 1 and 2 respectively. The control group was matched for age and sex, and as expected, hypertension (cases 58.4% vs. controls 16.8%), diabetes (cases 31.6% vs. controls 10.8%), and dyslipidemia (cases 22.8% vs. controls 5.6%) were found significantly more often in cases than in controls ($p < 0.05$).

All genotype and allelic frequencies were in HWE in both IS patients and controls. Genetic analysis for MTHFR C677T gene polymorphism was conducted for all 250 IS cases and 250 age and sex matched controls and is summarized in Table 3. Conditional logistic regression analysis showed an independent association of MTHFR C677T gene polymorphism with risk of IS even after adjusting the effects of potential confounding factors under dominant model [OR 1.91; 95% CI 1.07–3.41; $p = 0.028$] and allelic model [OR 1.44; 95% CI 1.03–2.02; $p = 0.03$] with the risk of IS.

In the subgroup analysis as per TOAST classification, an independent association was observed between MTHFR C677T polymorphism and risk of SVD, even after controlling

Table 1 Characteristics of ischemic stroke patients and controls.

S. No.	Characteristics	Ischemic stroke ($N = 250$)	Controls ($N = 250$)
1.	Age in years (Mean ± S.D)	52.83 ± 12.59	50.97 ± 12.70
2.	Male/female, *n*	203/47	203/47
3.	SBP, mmHg (Mean ± S.D)	136.58 ± 23.67	132.06 ± 19.43
4.	DBP, mmHg (Mean ± S.D)	81.68 ± 12.43	81.26 ± 10.74
5.	Random blood sugar (mg/dl) (Mean ± S.D)	119.44 ± 43.68	–
6.	Total cholesterol (mg/dl) (Mean ± S.D)	168.67 ± 51.42	–
7.	Triglycerides (mg/dl) (Mean ± S.D)	126.58 ± 64.33	–
8.	Previous stroke record, *n* (%)	39 (15.6)	–
9.	Stroke in young, *n* (%)	74 (29.6)	–

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 2 Demographic and risk factor variables for ischemic stroke (IS) patients and control subjects.

Characteristics	Controls ($N = 250$) n (%)	Ischemic stroke ($N = 250$) n (%)	Crude OR [95% CI], p value	*Adjusted OR [95% CI], p value
Age in years (Mean ± S.D)	52.83 ± 12.59	50.97 ± 12.70	**Matched**	
Male/female, n	203/47	203/47		
Hypertension	42 (16.8)	146 (58.4)	**8.4 [4.8–14.6], < 0.0001**	**6.2 [3.2–12], < 0.0001**
Diabetes	26 (10.4)	79 (31.6)	**3.5 [2.1–5.7], < 0.0001**	**2.1 [1.1–4.2], 0.02**
Dyslipidemia	14 (5.6)	57 (22.8)	**5.2 [2.6–10.4], < 0.0001**	**2.4 [1.0–5.7], 0.04**
Smoking	67 (26.8)	97 (38.8)	**1.7 [1.1–2.5], 0.005**	1.1 [0.6–1.9], 0.69
Alcohol	56 (22.4)	81 (32.4)	**1.8 [1.1–2.8], 0.008**	1.8 [0.9–3.5], 0.05
Myocardial infarction	4 (1.6)	17 (6.8)	**5.3 [1.5–18.3], 0.008**	1.8 [0.4–7.1], 0.36
Migraine with aura	8 (3.2)	10 (4)	1.2 [0.4–3.1], 0.63	1.6 [0.4–5.8], 0.40
Migraine without aura	4 (1.6)	5 (2)	1.2 [0.3–4.6], 0.33	1.3 [0.1–10.9], 0.78
Low socioeconomic status	14 (5.6)	66 (26.4)	**5.0 [2.7–9.0], < 0.0001**	**7.5 [3.0–18.2], < 0.0001**
High BMI	89 (35.6)	77 (30.8)	0.7 [0.4–1.0], 0.11	0.8 [0.4–1.4], 0.50
Sedentary life style	106 (42.4)	127 (50.8)	**1.4 [1.0–2.1], 0.04**	1.1 [0.6–1.9], 0.6
Physical activity	136 (54.4)	107 (42.8)	**0.6 [0.4–0.8], 0.009**	0.7 [0.4–1.3], 0.36
Family history of stroke	9 (3.6)	32 (12.8)	**3.5 [1.6–7.4], 0.001**	**6.8 [2.2–20.9], 0.001**
Family history of diabetes	28 (11.2)	48 (19.2)	**1.8 [1.1–3.1], 0.015**	**3.1 [1.4–6.9], 0.004**
Family history of hypertension	34 (13.6)	61 (24.4)	**2.0 [1.2–3.1], 0.003**	1.8 [0.9–3.4], 0.06
Family history of heart attack	14 (5.6)	19 (7.6)	1.4 [0.6–3.1], 0.33	1.7[0.5–5.9], 0.38

Abbreviations: IS, ischemic stroke; BMI, body mass index; OR, odds ratio; CI, confidence interval.
Conditional logistic regression analysis.
Bold values indicate the significance level of $p < 0.05$.
* Adjusted variables include hypertension, diabetes, dyslipidemia, smoking, family history of stroke, alcohol, sedentary life style and low socioeconomic status.

the effects of potential confounding in multivariable logistic regression analysis. [OR 2.51; 95% CI 1.30–4.85; $p = 0.006$]. A significant association was observed with cardioembolic (CE) [OR 2.34; 95% CI 1.03–5.31; $p = 0.042$] stroke under the dominant model of inheritance.

4. Discussion

The present case–control study demonstrated that the C677T polymorphism of MTHFR gene was significantly associated with the increased risk of IS in the North Indian population. Several studies have been conducted to determine the association between MTHFR C677T polymorphism and IS; however, the conclusion still remains controversial. Our results are consistent with the recently published meta-analysis which consisted of 38 studies involving 6310 cases and 8297 controls which found a significant association with the risk of IS [17]. Based on ethnicity, this meta-analysis suggests a significant association between MTHFR C677T gene polymorphism and IS risk in both the Asian as well as Caucasian populations; however, strength of association was higher in Asian as compared to the Caucasian population. A recent meta-analysis which included 68 case–control studies containing 7990 cases and 6941 controls from Chinese population also confirmed the significant association between MTHFR C677T polymorphism and risk of cerebrovascular diseases (OR 1.81, 95% CI 1.6–1.9: TT vs. CC) [26]. Our findings suggest that MTHFR C677T gene polymorphism may be used as a genetic marker for IS in North Indian population.

Although the exact mechanism by which MTHFR polymorphism affects IS has not yet been fully elucidated, some possible mechanisms have been put forward. Studies have shown that a single base pair replacement of Cytosine nucleotide with thymine at 677 nucleotide position of MTHFR gene influences enzyme thermolability, its decreased activity and, in turn, the elevated level of homocysteine, an endothelial toxin especially in the presence of low folate levels and in effect to elevated risk of stroke [27,28]. Elevated levels of Homocysteine may lead to endothelial dysfunction, an early stage for the initiation of atherosclerosis. A meta-analysis which involved 72 case–control studies revealed an odds ratio of 1.42 for 5 μmol/l and showed an increase in the homocysteine level. A meta-analysis suggested a mean difference in serum homocysteine between TT and CC genotype of 2.7 mmol/L [29]. Hyperhomocysteinemia is also linked to DNA hypomethylation in vascular diseases [30]. A study published by Castro et al. in 2004 demonstrated that MTHFR C677T gene polymorphism with concomitant inadequate folate level is associated with DNA hypomethylation and thereby increases the risk for atherosclerosis [31]. A study has shown that TT genotype at 677 position of MTHFR polymorphism is linked with diminished genomic DNA methylation as compared to those with wild type CC genotype (32.23 vs. 62.24 ng 5-methylcytosine/microg DNA, $p < 0.0001$) [32]. DNA hypomethylation is directly correlated with folate status and inversely with homocysteine level [32]. An Asian study demonstrated that CpG methylation in MTHFR is significantly associated with 4.74 increased susceptibility of IS by mediating serum folate and vitamin B-12 level [16]. Other studies have shown lower folate levels in patients with myocardial infarction and coronary artery disease [6,19–20]. Vitamin intervention may bring down the higher level of Hcy [33,34]. A meta-analysis suggested that folic acid supplementation could significantly reduce the risk of stroke by 18% [35]. These findings explain the mechanism of association between MTHFR polymorphism and pathogenesis of IS.

Table 3 Genotype and allelic frequencies of MTHFR C677T gene polymorphisms in IS patients and controls.

Polymorphisms			LVD $N = 107$	SVD $N = 83$	CE $N = 26$	Others $N = 34$	IS $N = 250$	Controls $N = 250$
C677T	Genotype	CC, *n* (%)	73 (68.23)	51 (61.45)	14 (53.85)	23 (67.65)	161 (64.4)	183 (73.2)
		CT, *n* (%)	32 (29.90)	31 (37.35)	11 (42.30)	10 (29.41)	84 (33.6)	65 (26)
		TT, *n* (%)	2 (1.87)	1 (1.20)	1 (3.85)	1 (2.94)	5 (2)	2 (0.8)
	Allele	C, *n* (%)	178 (83.18)	133 (80.12)	39 (75)	56 (82.35)	406 (81.2)	431 (86.2)
		T, *n* (%)	36 (16.82)	33 (19.88)	13 (25)	12 (17.65)	94 (18.8)	69 (13.8)
	Dominant CT + TT vs. CC	Adjusted OR (95% CI), *p* value	1.31 (0.70–2.44), 0.38	**2.51 (1.30–4.85), 0.006**	2.42 (0.87–6.71), 0.08	1.28 (0.51–3.21), 0.59	**1.91 (1.07–3.41), 0.028**	
		Unadjusted OR (95% CI), *p* value	1.27 (0.77–2.08), 0.33	**1.71 (1.01–2.89), 0.04**	**2.34 (1.03–5.31), 0.042**	1.30 (0.60–2.82), 0.49	**1.55 (1.04–2.30), 0.03**	
	Recessive TT vs. CT + CC	Adjusted OR (95% CI), *p* value	2.21 (0.18–26.09), 0.52	1.27 (0.06–27.08), 0.87	NE	NE	1.86 (0.18–18.91), 0.59	
		Unadjusted OR (95% CI), *p* value	2.36 (0.32–16.99), 0.39	1.51 (0.13–16.89), 0.73	4.96 (0.43–56.64), 0.19	3.75 (0.33–42.58), 0.28	2.50 (0.48–12.88), 0.27	
	Allelic C vs. T	OR (95% CI), *p* value	1.26 (0.81–1.96), 0.29	1.54 (0.98–2.45), 0.06	2.08 (1.05–4.09), 0.03	1.33 (0.68–2.62), 0.39	1.44 (1.03–2.02), 0.03	

Abbreviations: C, cytosine; CI, confidence interval; T, thymine; IS, ischemic stroke; LVD, large vessel disease; SVD, small vessel disease; CE, cardio embolic; OR, odds ratio; NE, not estimable (the confidence interval could not be estimated).

Bold values indicate the significance level of $p < 0.05$.

*$p < 0.05$ is considered as statistically significant.

**Adjusted analysis was done by adjusting hypertension, diabetes, dyslipidemia, smoking, family stroke record, alcohol, sedentary life style, low economic status variables.

Literature suggests that susceptibility of IS risk varies in different subtypes of IS [36]. Our stratified analysis based on TOAST classification suggested an independent association between MTHFR C677T gene polymorphism and risk of SVD subtype of IS. A study also observed that MTHFR C677T polymorphism is associated with multiple small artery occlusion [37].

There were a few limitations in our study. First, the study was conducted in a single hospital and the participants might not have been the representatives from other areas. Therefore, further large sample size and multicentric studies are needed to confirm our findings. Second, we selected single SNP (rs1801133) as emerging evidences have shown that the C/T substitution is responsible for regulating the Hcy levels. Third, we did not collect data for blood sugar, cholesterol, triglycerides under controls and this study did not estimate the hcy levels. Despite these limitations, our study provides strong evidence for the independent association between MTHFR C677T gene polymorphism and risk of IS in the North Indian population.

5. Conclusion

In summary, the findings of the present case–control study suggest that polymorphism in C677T position of MTHFR gene might be a risk factor for IS in North Indian population.

Conflict of interest

The authors have declared that no competing interests exist.

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